

Immuno-EM analysis of PF13_0191-GFP Expressing Parasites

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[Abstract] This protocol was used to prepare pre-embedding samples of *Plasmodium falciparum* blood stage parasites that overexpressed the parasite protein PF13_0191 tagged with GFP. Using GFP-specific antibodies and Protein A-Gold the localisation of the overexpressed protein in the infected host cell was determined using standard transmission electron microscopy (EM). Pre-embedding EM is a common method where the antibodies are introduced before embedding and sectioning. This method avoids the problem that antigens are often difficult to detect on EM-sections after embedding. In the method described here antigens in the parasite-infected host cell are detected. Entry of the antibody is made possible through permeabilisation of the host cell with tetanolysin. In principle this method could also be used to detect antigens within the parasite if the sample is appropriately fixed and permeabilised before addition of the relevant antibody. While access of the antibody will avoid the detection problems often seen with post-embedding methods, this procedure will produce comparably poorer morphology.

Materials and Reagents

A. Parasite culture

1. *Plasmodium falciparum* wildtype 3D7 parasites transfected with your protein of interest tagged with GFP
2. Sterile, human 0⁺ erythrocyte concentrate (Blood Banking)
3. RPMI complete medium (see Recipes)
 - a. RPMI-1640 (AppliChem GmbH, catalog number: A1538,9010)
 - b. NaHCO₃ (Sigma-Aldrich, catalog number: S5761)
 - c. Glucose (Merck KgaA, catalog number: 1.08342.1000)
 - d. AlbumaxII (Life Technologies, Gibco[®], catalog number: 11021-037)
 - e. Hypoxanthine (Sigma-Aldrich, catalog number: H9636)
 - f. Gentamicin (Ratiopharm GmbH)

B. Pre-embedding samples

1. Percoll (GE Healthcare, catalog number: 17-0891-02)
2. Formaldehyde (Polysciences)

3. Tetanolysin (List Biological Labs, catalog number: 199)
4. BSA (Enzo Life Sciences, Biomol[®])
5. Mouse anti-GFP (Roche Diagnostics, catalog number: 11814460001)
6. Rabbit anti-mouse linker antibodies (Dako, catalog number: Z0259)
7. Protein A-Gold (6 nm) (Aurion, catalog number: 806-111)
8. 25 % Glutaraldehyde (Electron Microscopy Sciences, catalog number: 16210)
9. OsO₄ (Electron Microscopy Sciences, catalog number: 19152)
10. Low melting agarose (Electron Microscopy Sciences, catalog number: 10207)
11. 100 % ethanol (Merck KGaA)
12. Propylene oxide (Science Services GmbH, catalog number: UN1280)
13. Gelatine capsules (Plano, catalog number: G29212)
14. Uranyl acetate (Agar Scientific, catalog number: R1260)
15. Methyl nadic anhydride (MNA) (Agar Scientific, catalog number: R1083)
16. Dodecenylsuccinic anhydride (DDSA) (Agar Scientific, catalog number: R1053)
17. EPON (Agar Scientific, catalog number: R1045)
18. Benzyltrimethylamine (BDMA) (Agar Scientific, catalog number: R1062b)
19. Pb(NO₃)₂ (Merck KGaA, catalog number: 12438)
20. Na(CH₃)₂AsO₂·3H₂O (Fluca)
21. Saccharose (Merck KGaA)
22. 10x PBS (see Recipes)
23. Tetanolysin (see Recipes)
24. Sodium cacodylate buffer (see Recipes)
25. EPON (see Recipes)
26. Lead citrate (pH 12) (Reynolds' stain) (see Recipes)

Equipment

1. Falcon tubes (15 ml, 50 ml)
2. Centrifuge
3. Eppendorf tubes (1.5 ml, 2 ml)
4. Sterilisation filters (0.22 µm)
5. Bunsen burner
6. Water bath
7. Glass bottles
8. Thermo block
9. Rolling device
10. Razor blades

11. Sealable glass vials
12. Tweezers
13. Ultramicrotome (Leica Microsystems)
14. Copper grids (Plano)
15. Transmission electron microscope (FEI)

Procedure

A. Preparation of pre-embedding samples

1. Centrifuge 4 x 10 ml of a *Plasmodium falciparum* culture (5-10% trophozoites and schizonts) in 15 ml falcon tubes at 500 x g for 5 min, discard the supernatant and resuspend the pellet in 4 x 10 ml 1 x PBS. Centrifuge again.
2. Purify infected red blood cells from culture using a Percoll gradient as described in Bio-protocol [Immuno-EM analysis of PF13 0191-GFP Expressing Parasites](#) (steps A2a-e) (Heiber and Spielmann, 2014).

Note: Combine the 4 pellets after the first washing step into one 1.5 ml Eppendorf tube.

3. Resuspend the pellet in 1 ml 1x PBS containing 2% formaldehyde and incubate at room temperature for 10 min on a rolling device.
4. Centrifuge at 300 x g for 3 min and wash the pellet 3x in 1 ml 1x PBS.
5. Resuspend the pellet in 800 µl 1x PBS, add 8 µl tetanolysin (1 µg/µl) and mix the solution immediately by flipping the tube with your finger. Incubate at 37 °C for 30 min and prevent the cells from sinking to the bottom by flipping the tube with your finger every few minutes.
6. Centrifuge at 300 x g for 3 min and wash the pellet once by resuspending it in 1 ml 1x PBS followed by centrifuge at 300 x g for 3 min.
7. Resuspend the pellet again in 1 ml 1x PBS containing 2% formaldehyde and incubate at room temperature for 5 min on a rolling device.
8. Centrifuge at 300 x g for 3 min and wash the pellet once by resuspending it in 1 ml 1x PBS followed by centrifuge at 300 x g for 3 min.
9. Resuspend the pellet in 500 µl 1x PBS containing 3% BSA and incubate at room temperature for 15 min on a rolling device.
10. Centrifuge at 300 x g for 3 min. Resuspend the pellet in 200 µl of freshly prepared 1:20 dilution of mouse anti-GFP antibody in 3 % BSA in 1x PBS and incubate at room temperature for 1.5 h on a rolling device.
11. Centrifuge at 300 x g for 3 min and wash the pellet twice in 1 ml 1x PBS.

12. Resuspend the pellet in 200 μ l of freshly prepared 1:25 dilution of rabbit anti-mouse linker antibody in 3% BSA in 1x PBS and incubate at room temperature for 1.5 h on a rolling device.
13. Centrifuge at 300 x g for 3 min and wash the pellet twice in 1 ml 1x PBS.
14. Resuspend the pellet in 200 μ l of freshly prepared 1:20 dilution of protein A gold in 3% BSA in 1x PBS and incubate at room temperature for 1.5 h on a rolling device.
15. Centrifuge at 300 x g for 3 min and wash the pellet twice in 1 ml 1x PBS.

B. Fixation

1. Resuspend the cells in 500 μ l sodium cacodylate buffer and add glutaraldehyde to 2% and incubate for 1 h at 4 °C.
Note: The sample can be stored at 4 °C until use.
2. Centrifuge at 6,000 x g for 3 min and wash the cells 3x with 500 μ l sodium cacodylate buffer.
3. Post fix the cells with 1% OsO₄ in cacodylate buffer for 30 min at 4 °C.
4. Centrifuge at 6,000 x g for 3 min and wash the cells 3x with 500 μ l sodium cacodylate buffer.
5. Prepare 3% low melting agarose by heating it over a Bunsen burner and cool down to 40 °C in a water bath.
6. Resuspend the cells in the 40 °C liquid 3% low melting agarose and in a 1.5 ml Eppendorf tube spin the cells down immediately at 13,000 x g for 5 min. Let the agarose harden for 30 min at 4 °C to form a stable agarose pellet containing the infected red blood cells.
7. Crop off the tip of the tube with a razor blade and remove the agarose pellet.

C. Dehydration of the sample through increasing concentrations of ethanol

1. Start with 30% ethanol and incubate for 10 min. Continue by transferring the sample to glass bottles containing 50%, 70%, 80%, 90%, respectively and 3x to a new bottle with 100% ethanol and incubate each time for 10 min.
2. Remove the agarose pellet from the ethanol and trim the embedded infected red blood cells with a razor blade into very small pieces (max. length of the edge ~1 mm).
3. Incubate the sample 2x for 10 min in propylene oxide to fully cover the sample.

D. Embedding and sectioning

Note: All steps have to be performed in glass vials since the propylene oxide dissolves the plastic tubes and sufficient volumes need to be used to fully cover the sample.

1. Incubate the sample in 3:1 propylene oxide/epon resin for 15 min in a sealed glass vial.

2. Incubate the sample in 1:1 propylene oxide/epon resin for 30 min in a sealed glass vial.
3. Incubate the sample in 1:3 propylene oxide/epon resin overnight in a sealed glass vial.
4. Replace the resin mixture with fresh pure epon resin and incubate in an unsealed glass vial for 2 h at room temperature.
5. Incubate for ~15 min at 60 °C to increase the fluidity of the resin.
6. Fill gelatine capsules with resin and add the sample with tweezers. Incubate the samples over night at 60 °C to harden the resin.
Note: The sample will sink to the bottom of the capsule.
7. Prepare 50 to 70 nm ultrathin sections with an ultramicrotome and place them on copper grids using tweezers.
8. Contrast the thin sections by placing them on a drop of 2% uranyl acetate for 5 min, followed by thorough rinsing in dH₂O. Then place sections on a drop of lead citrate for 5 min, rinse thoroughly in dH₂O and view the samples in a transmission electron microscope at 80 kV. An example for the appearance of infected RBCs in a section prepared according to the pre-embedding method is shown in Figure 1.

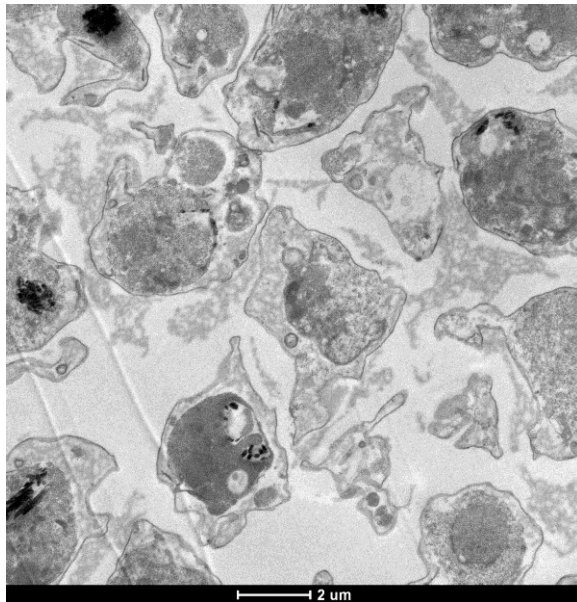


Figure 1. Example of a section of the pre-embedded parasite material. Size bar 2 μm

Recipes

1. RPMI complete medium (1 L)
 - 15.87 g RPMI-1640
 - 1 g NaHCO₃
 - 2 g glucose

- 5 g Albumax II
 0.0272 g hypoxanthine
 0.5 ml gentamicin (40 mg/ml, Ratiopharm)
 Adjust pH to 7.2 using HCl, 1 L dH₂O
 Filter sterilize (0.22 μm) and stored at 4 °C
2. 10x PBS (1 L)
 5.7 g Na₂HPO₄
 1.25 g NaH₂PO₄
 15.2 g NaCl
 Adjust pH to pH 7.4, 1 L dH₂O
3. Tetanolysin
 Dissolved at 1 μg/μl in dH₂O
 Make aliquots and store at -20 °C
4. Sodium cacodylate buffer (0.5 L, 0.1 M, pH 7.2)
 10.7 g Na(CH₃)₂AsO₂·3H₂O
 17.12 g saccharose
 0.111 g CaCl₂
 460 ml dH₂O
 40 ml 0.1 M HCl
5. EPON
 13.4 g MNA
 10.78 g DDSA
 25.82 g EPON
 Agitate for 30 min
 Add 1.5% BDMA
 Agitate for 5 min
 Stored at -20 °C
6. Lead citrate (pH 12) (Reynolds' stain)
 1.33 g Pb(NO₃)₂
 1.76 g tri-Natriumcitrate·2H₂O
 17.12 g saccharose
 30 ml H₂O
 Agitate for 1 min and incubate for 30 min
 Add 8 ml NaOH (Solution has to be clear.)
 Add H₂O to 50 ml and stored in a sealed glass container (the solution is stable for 6 months.)
 For use dilute 1:10 in 0.1 M NaOH

Acknowledgments

We gratefully acknowledge the work from the Tilley lab that developed the method for pre-embedding EM in selectively permeabilised *P. falciparum*-infected cells that forms the basis for the procedures described here: Jackson *et al.* (2007). The Percoll gradient is a modified version of the protocol from Aley *et al.* (1986). The Percoll purification is described in more detail in the Bio-protocol 'Preparation of parasite protein extracts and Western blot analysis' (Procedure 1.2.a-e).

References

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